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# Modulation of Tumor Cell Proliferation and Apoptosis by Polyamine Depletion in Cells of Head and Neck Squamous Cell Carcinomas

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**Bock, J. M., Pickart, M. A., Pink, J. J. and Harari, P. M. Modulation of Tumor Cell Proliferation and Apoptosis by Polyamine Depletion in Cells of Head and Neck Squamous Cell Carcinomas. *Radiat. Res.* 152, 604–610 (1999).**

**These studies were carried out to examine the capacity of α-difluoromethylornithine (DFMO) to modulate cell proliferation and apoptosis in cells of squamous cell carcinomas (SCCs) of the head and neck. Exposure of cells to DFMO (5 m*M* for 48 h) depleted intracellular putrescine and spermidine levels (greater than 5-fold) and inhibited proliferation of the cells without manifestation of cytotoxicity as measured by a clonogenic assay. Exposure of the cells to DFMO did not influence the survival response after exposure to single-dose radiation between 0 and 10 Gy. Treatment of polyamine-depleted cells with 200 n*M* staurosporine amplified apoptosis 65% (1.65-fold) over that in controls, as determined by flow cytometry. The increased apoptosis after DFMO treatment was effectively inhibited by the addition of 1 m*M* putrescine or spermidine. Cleavage of poly(ADP-ribose) polymerase (PARP) illustrated that the staurosporine treatment induced apoptosis in the cells within 6 h. Analysis of PARP cleavage indicated that treatment with DFMO accelerated the kinetics of progression of apoptosis but did not influence the sensitivity of cells to 10 n*M*–1 μ*M* staurosporine. These data suggest an involvement of endogenous polyamines in modulation of proliferation kinetics and apoptosis in human SCCs and suggest opportunities to explore new therapeutic strategies in head and neck cancer patients to be treated with radiation therapy.** 

## INTRODUCTION

Our advancing understanding of apoptosis (*1*) has fostered new insight regarding the normal characteristics of tumor growth and the response of cells to cancer therapy. In some systems, a delicate balance exists between cell proliferation and apoptosis in the natural history of malignant growth (*2*). Squamous cell carcinomas (SCCs) of the head and neck are among the most rapidly proliferating human solid tumors, with mean potential doubling times *in situ* of 3–5 days. This high proliferative capacity affords SCCs the ability to repopulate during traditional cytotoxic therapy, thereby increasing the overall clonogenic burden that must be eradicated to effect cure (*3*). Investigation of methods to inhibit the proliferation of these tumor cells during therapy (*4*), or to promote apoptosis (*5*), represents approaches that may favorably influence the outcome of therapy for patients with head and neck SCCs. The endogenous intracellular polyamines represent a potential target of interest because of their capacity to modulate the balance between tumor cell proliferation and apoptosis (*4, 6*).

The capacity of endogenous polyamines to influence cell proliferation and growth kinetics in mammalian cells is well established (*6*), and information about the influence of polyamines on the initiation and progression of apoptosis is emerging. Treatment of thymocytes with spermine inhibits its activation of apoptosis-associated nuclear endonucleases and formation of DNA ladders (*7*). Parchment has suggested that the reactive oxygen species formed during the enzymatic oxidation of polyamines may play a role in the production of apoptosis during the development of embryonic cells (*8*). The *MYC* oncogene is implicated as an inducer of apoptosis through transcriptional control of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis; difluoromethylornithine (DFMO) slows this induction (*9*). This work examines the relationship between endogenous polyamines, cell proliferation, and apoptosis in cells of rapidly proliferating SCCs of the head and neck.

## MATERIALS AND METHODS

### *Drugs*

α-Difluoromethylornithine was generously provided by Ilex Oncology, Inc., San Antonio, TX. Stock solutions of DFMO and putrescine (1 *M*) were dissolved in water and filter-sterilized. Spermidine solutions (1 *M*) were prepared in supplemented medium in the presence of 1 m*M* aminoguanidine to quench serum oxidases. Stock solutions of staurosporine (500 μ*M*) were prepared in 100% ethanol. Stock solutions of camptothecin (5 m*M*) were prepared in dimethylsulfoxide.

### *Tissue Culture and Cell Lines*

The human SCC-13Y and SCC-4Y cell lines were originally established from biopsies from head and neck cancer patients and were generously provided by Dr. B. Lynn Allen-Hoffman (University of Wisconsin). The cells were maintained as monolayer cultures as reported previously (*4*). Briefly, cell cultures were maintained in Dulbecco's modified Eagle's medium (Gib-

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co) supplemented with 10% Fetal Clone II serum, 1% penicillin-streptomycin, and 0.8 μg/ml hydrocortisone. Cell density and viability were measured by a hemocytometer and trypan blue dye exclusion.

*Phase and Fluorescence Microscopy Assays*

Cells were grown on glass cover slips and fixed in 4% paraformaldehyde. After RNase and proteinase K treatment, cells were stained with hematoxylin and Hoechst 33258. After dehydration, cover slips were mounted on slides and examined with high-resolution fluorescence microscopy. Apoptotic nuclei were identified by visualization of chromatin condensation by Hoechst staining.

*Polyamine Analysis*

Separation of the natural polyamines and their monoacetyl derivatives was achieved by reverse-phase high-performance liquid chromatography (HPLC) using modifications of an established method (*10*). The quantitative analyses reported herein were performed in triplicate and reported as the mean ± SEM. Absolute polyamine levels are expressed as nmol/mg protein or as a percentage of polyamine depletion compared to control samples.

*Radiation Survival*

Exponentially growing cultures of SCC-4Y and SCC-13Y cells were irradiated in T-25 culture flasks with single exposures at the doses specified. Cells were subsequently trypsinized, counted, serially diluted, and plated in triplicate for colony formation. Scored colonies were defined as containing greater than or equal to 50 cells. After an incubation interval of 14–21 days, colonies were stained with crystal violet and counted manually. Mean survival values from the triplicate samples were calculated and plotted ± SEM and are shown as bar graphs.

*PARP Cleavage and Western Blotting*

Samples were probed to detect native PARP and cleavage products by harvesting preconfluent cells from the medium and monolayer in cold phosphate-buffered saline (PBS; 150 m*M* NaCl, 50 m*M* $KH_2PO_4$, 45 m*M* KOH). After centrifugation, cell pellets were resuspended in PARP-lysis buffer [62.5 m*M* Tris-Cl, pH 6.8, 6 *M* urea, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 0.003% bromophenol blue] and sonicated on ice for 30 s (Fisher Scientific 550 Sonic Dismembrator) SDS-polyacrylamide gel electrophoresis and Western blot transfer allowed separation of sample proteins. The transfer membrane was stained with Ponceau S to check for equal protein loading and then blocked for 1 h in PBS/10% FBS/0.1% Tween/1 m*M* sodium azide. Immunodetection of PARP cleavage was performed by incubation overnight with the C2-10 anti-PARP antibody (1:5000 dilution, Enzyme System Products, Dublin, CA) followed by 2 h incubation with 1:2000 dilution of secondary anti-mouse IgG horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). PARP cleavage was detected via chemiluminescence visualization with ECL (Amersham Life Sciences, Arlington Heights, IL). Quantification of PARP cleavage was performed using a Gel Doc 1000 (Bio-Rad, Hercules, CA) as described previously (*11*). Briefly, equal areas from each lane were scanned and the signal was integrated. Background areas were subtracted from each area and the percentage of signal at 89 kDa was calculated by dividing the 89-kDa signal by the total signal at 113 and 89 kDa.

*Quantification of Apoptosis by Flow Cytometry*

Detection of apoptosis by flow cytometry was performed using a modification of an established protocol (*12*). Cells were harvested with trypsin and counted using a hemocytometer and trypan blue exclusion. Samples were centrifuged and fixed in 900 μl ice-cold 95% ethanol plus 100 μl cold PBS for 24 h. After resuspension in phosphate-citric acid buffer (0.192 m*M* $Na_2HPO_4$, 4 m*M* citric acid and 1 m*M* EDTA) and PBS for 45 min, the cells were stained overnight with PI staining solution [0.33 mg/ml RNase A, 33 μg/ml PI, 0.5% (v/v) Triton X-100, 1 m*M* EDTA in PBS]. Samples were filtered and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Flow cytometry analyses were evaluated from histogram displays depicting total DNA content and fitted for extrapolation of apoptosis (ModFit LT software, Verity Software House, Inc., Topsham, ME). All experiments were performed in triplicate with errors calculated as SEM.

## RESULTS

### *DFMO-Induced Polyamine Depletion and Growth Inhibition*

High-performance liquid chromatography analysis of DFMO-treated cells (5 m*M* × 48 h) demonstrated depletion of endogenous putrescine and spermidine to essentially undetectable levels compared to controls (Fig. 1A). As shown previously (*4, 13*), exposure to DFMO exerted little effect on intracellular spermine levels. Analysis of intracellular polyamine levels in STS-treated cells showed that STS treatment alone did not perturb intracellular polyamine pools (data not shown). HPLC analyses of polyamines were repeated in triplicate, and errors were computed as SEM. Exposure of all tested SCC cells to DFMO (5 m*M*) resulted in marked growth inhibition by 3–4 days. Figure 1B provides a representative growth inhibition profile in SCC-13Y cells. Clonogenic survival assays demonstrated that exposure to 5 m*M* DFMO for 48 h induced no appreciable cytotoxicity in these cells (data not shown).

### *Radiation Survival*

The influence of treatment with DFMO (5 m*M* for 48–72 h) on single-dose survival was examined in the SCC-4Y and SCC-13Y cells as depicted in Fig. 2. Despite minor differences in the characteristic radiation profile for each cell line, no differences were observed with regard to the presence or absence of DFMO exposure on the radiation survival response.

### *Analysis of Apoptosis by Phase and Fluorescence Microscopy*

Microscopic analysis of STS-treated cells was performed to examine apoptosis in the human SCC cells. Morphological changes characteristic of apoptosis (*1*) were identified, including chromatin condensation, cytoplasmic blebbing, and perinuclear staining. Confirmation of these morphological changes was made by demonstration of chromatin condensation in STS-treated cells using Hoechst 33258 staining (Fig. 3). Treatment with DFMO and the resultant polyamine depletion produced low levels of chromatin condensation that were similar to those observed in controls.

### *Quantification of Apoptosis by Flow Cytometry*

Treatment with 200 n*M* STS reproducibly induced apoptosis (Fig. 4A and B), as did treatment with 5 μ*M* camptothecin (data not shown). To examine the effect of polyamine depletion on STS-induced apoptosis, SCC-13Y and SCC-4Y cells were incubated with 5 m*M* DFMO for 48 h prior to treatment with STS. Polyamine-depleted cells were

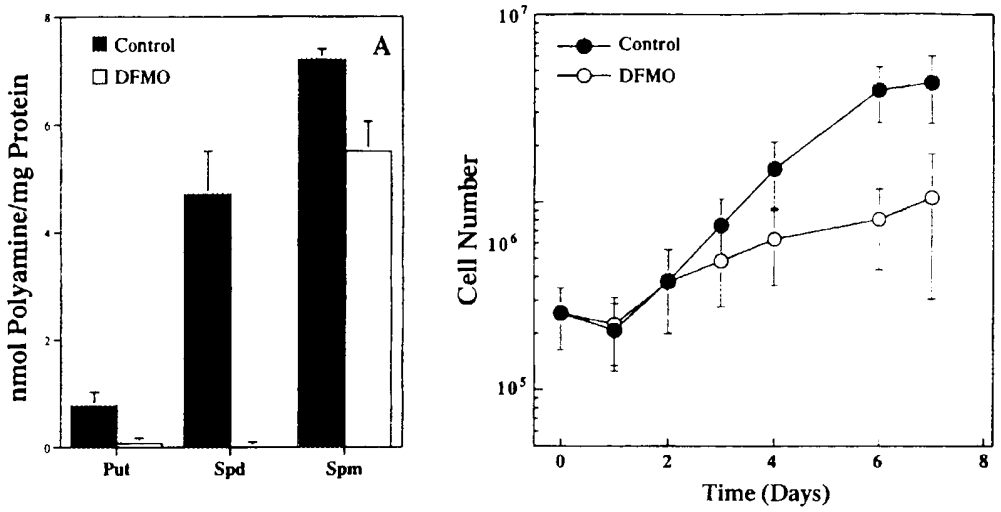



**FIG. 1.** Polyamine depletion and growth inhibitory effects of DFMO. SCC-13Y cells were exposed to DFMO (5 m*M* × 48 h) prior to harvesting for quantification of endogenous polyamine levels by HPLC as depicted in panel A (Put = putrescine, Spd = spermidine, Spm = spermine). Growth curve profiles for SCC-13Y cells after DFMO exposure (5 m*M* × 72 h present from day 0–3) are shown in panel B. Error bars for both panels represent the mean ± SEM for replicate experiments.

then treated with 200 n*M* STS for 8 h in the presence of 5 m*M* DFMO and assayed for percentage apoptosis by extrapolation of sub-$G_1$-phase DNA content as determined by PI staining. The polyamine-depleted cells produced 65% (1.65-fold) higher levels of apoptosis than cells treated with STS alone. When DFMO-treated SCC-13Y cells were incubated in the presence of 1 m*M* putrescine, the amplification of apoptosis was abolished. Treatment with 1 m*M* spermidine produced similar results. Treatment with 5 m*M* DFMO alone, 1 m*M* aminoguanidine alone, or 1 m*M* putrescine alone yielded levels of apoptosis indistinguishable from that observed in control cells within experimental error (approximately 3%).

*Effects of Polyamine Depletion on PARP Cleavage*

SCC-13Y cells were treated with STS for 8 h and assayed for PARP cleavage. The cells reproducibly demonstrated PARP cleavage after treatment with a range of STS concentrations (Fig. 5A). Polyamine depletion accelerated the rate of formation of the $M_r$ 89-kDa PARP fragment compared to control cells after treatment with 200 n*M* STS (Fig. 5B). To examine the effect of polyamine depletion on the sensitivity of SCC-13Y cells to STS, the cells were incubated for 8 h in STS at concentrations ranging from 50 p*M* to 1 μ*M*. STS induced cleavage of native PARP at all concentrations tested.

## DISCUSSION

The antiproliferative effect of polyamine depletion holds promise both as a chemopreventive agent (*14*) and as a potential adjuvant to conventional radiotherapy and/or chemotherapy in rapidly dividing tumors (*4*). To complement the established antiproliferative effects of polyamine deple-

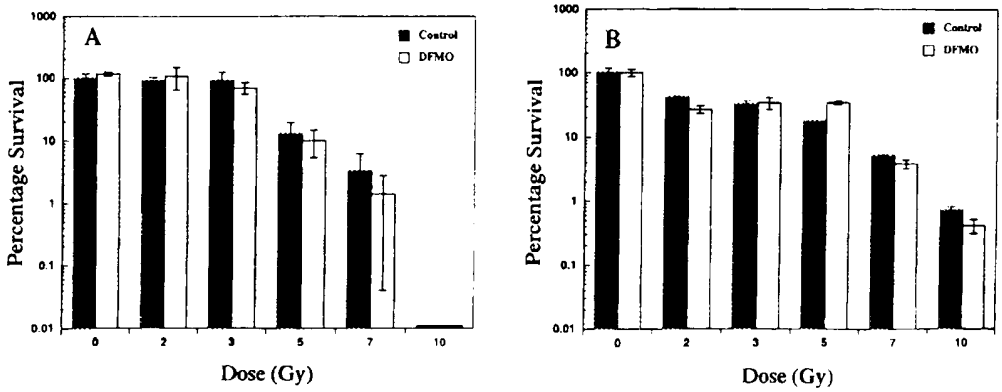



**FIG. 2.** Radiation survival. Lack of effect of 5 m*M* DFMO for 48–72 h prior to irradiation on *in vitro* radiosensitivity (single-dose survival). Panel A represents SCC-4Y cells, and Panel B represents SCC-13Y cells.

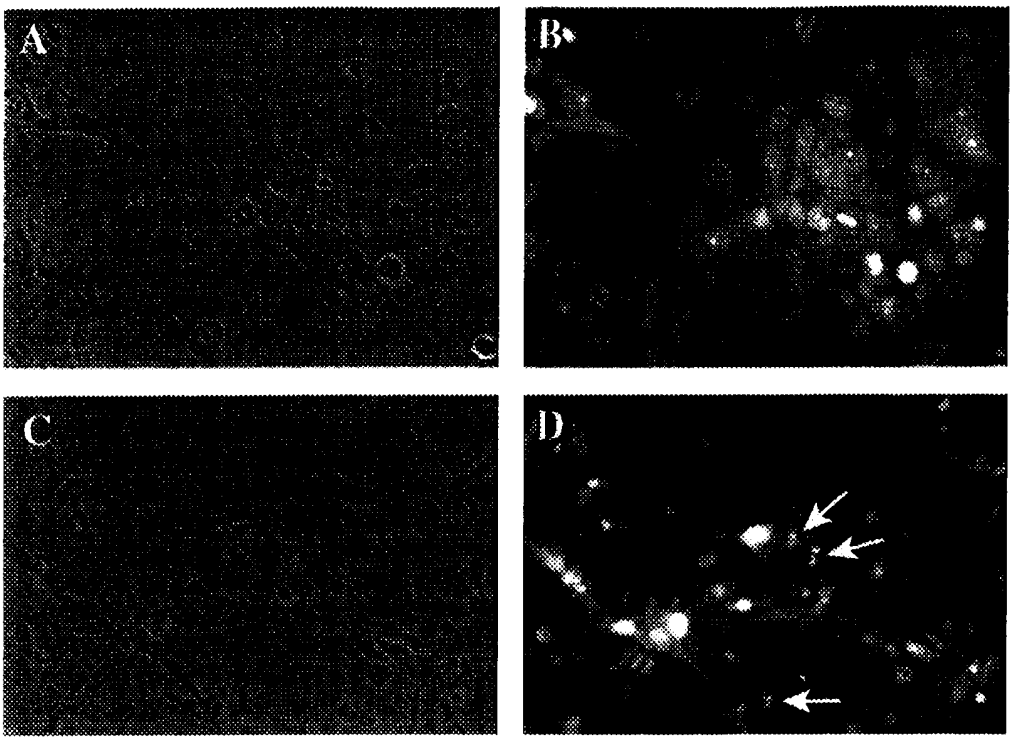
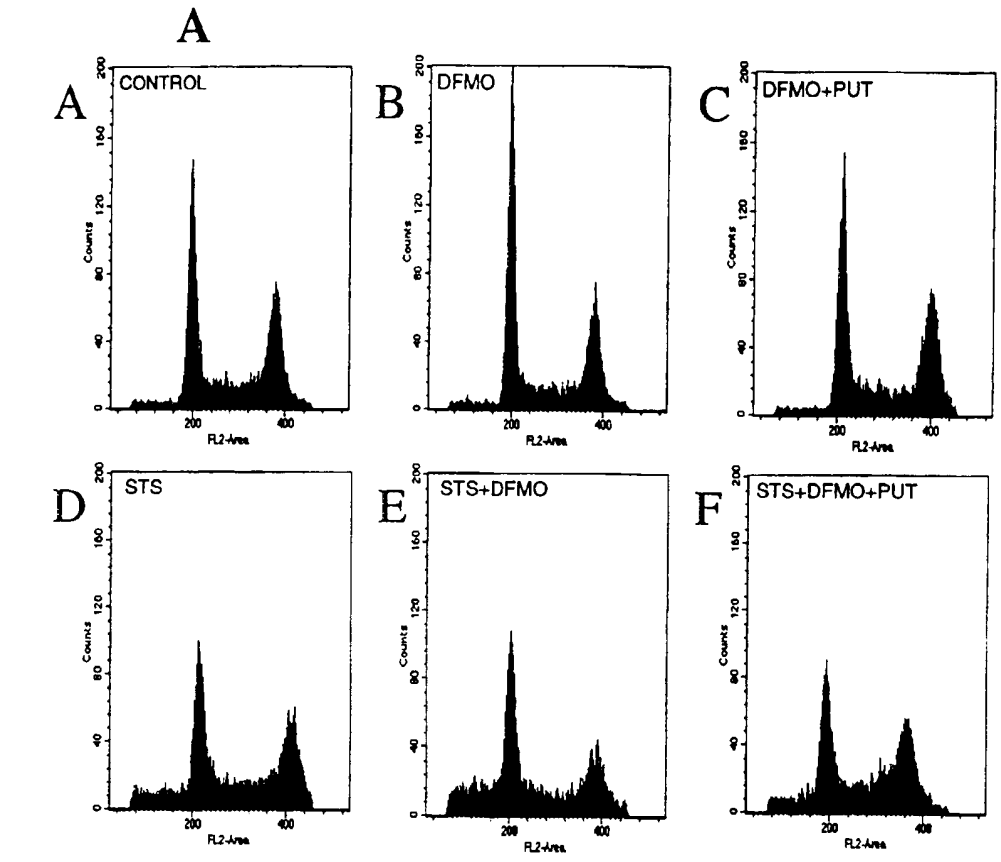


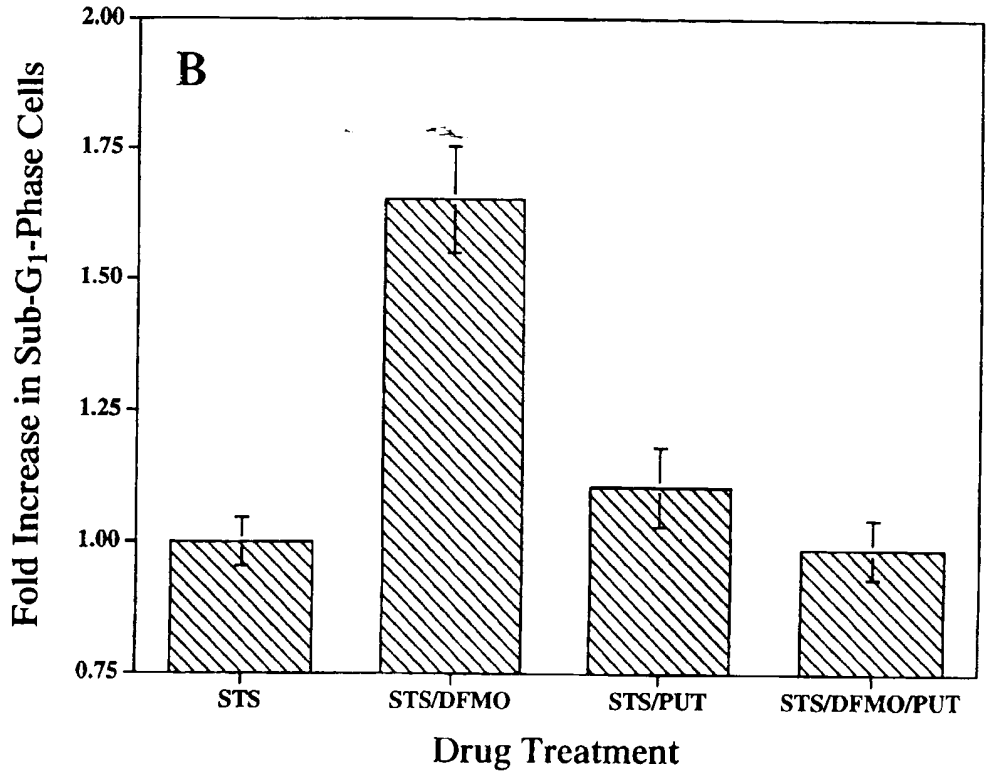

FIG. 3. Microscopic analysis of apoptosis in SCC cells. Morphological characteristics of SCC-13Y cells after exposure to 200 n*M* staurosporine (STS). Control (panel A) and STS-treated cells (panel C) visualized by phase microscopy (original magnification 400×). Control (panel B) and STS-treated cells (panel D) visualized by fluorescence microscopy after staining with 5 μg/ml Hoechst 33258 (original magnification 400×). Arrows in panel D illustrate nuclear condensation and fragmentation in apoptotic cells.

tion, we examined the influence of polyamine depletion in modulating apoptosis in cells of human SCCs. The ability of exogenously added polyamines to inhibit DNA laddering and endonuclease activation, presumably by protection of DNA through polyamine binding, has been identified previously (*7, 15*). The results above, which demonstrate that DFMO-induced polyamine depletion induces a 65% amplification of apoptosis in cells of human SCCs, further suggest that endogenous polyamines can modulate the level of apoptosis. The capacity of polyamine depletion to slow proliferation in rapidly dividing tumors and to induce amplification of apoptosis suggests a potential strategy for the treatment of human SCCs. Slowing of tumor cell proliferation in conjunction with amplification of apoptosis could serve to decrease the clonogenic burden requiring sterilization by conventional cytotoxic therapy in patients with rapidly proliferating SCCs of the head and neck.

In this study, amplification of apoptosis was consistently observed in SCC cells treated with STS (as determined by flow cytometry and PARP cleavage), whereas polyamine depletion produced minimal changes in camptothecin-induced apoptosis (data not shown). The specificity of this result to STS-induced apoptosis may be related to the cell cycle phase in which the inducing agents are most active. Lower concentrations of STS are most active in $G_1$ phase of the cell cycle (*16*). DFMO induces a transient cell cycle block in cells of SCCs in $G_1$ phase (Harari, unpublished data), and ornithine decarboxylase activity is necessary for progression into S phase (*17*). Therefore, similar timing of action within the $G_1$ phase of the cell cycle may be responsible for the ability of DFMO to amplify STS-induced apoptosis in head and neck SCCs. Inducers of apoptosis that are most active in other cell cycle phases, such as the S-phase inducer camptothecin, may therefore be less effective at inducing apoptosis after polyamine depletion.

The mechanisms whereby endogenous polyamines influence the initiation and progression of apoptosis remain to be clarified. Evidence of an interaction between polyamines and apoptosis emerged from studies in developmental biology, showing that by-products of polyamine catabolism (reactive oxygen species) are requisite for the development of embryonic cells (*8*). The importance of polyamine catabolism products and reactive oxygen species in the propagation of apoptosis is controversial (*18, 19*). Variation in intracellular polyamine levels alone may be sufficient to induce apoptosis (*20*). Under selected conditions, however, DFMO-induced polyamine depletion appears to suppress apoptosis. DFMO suppresses apoptosis in HeLa-TV cells by inhibition of tissue transglutaminase (*21*) and delays the progression of *MYC*-induced apoptosis in cells overexpressing ornithine decarboxylase (*9*). These variations may be due to differences in cell proliferation kinetics. Highly pro-



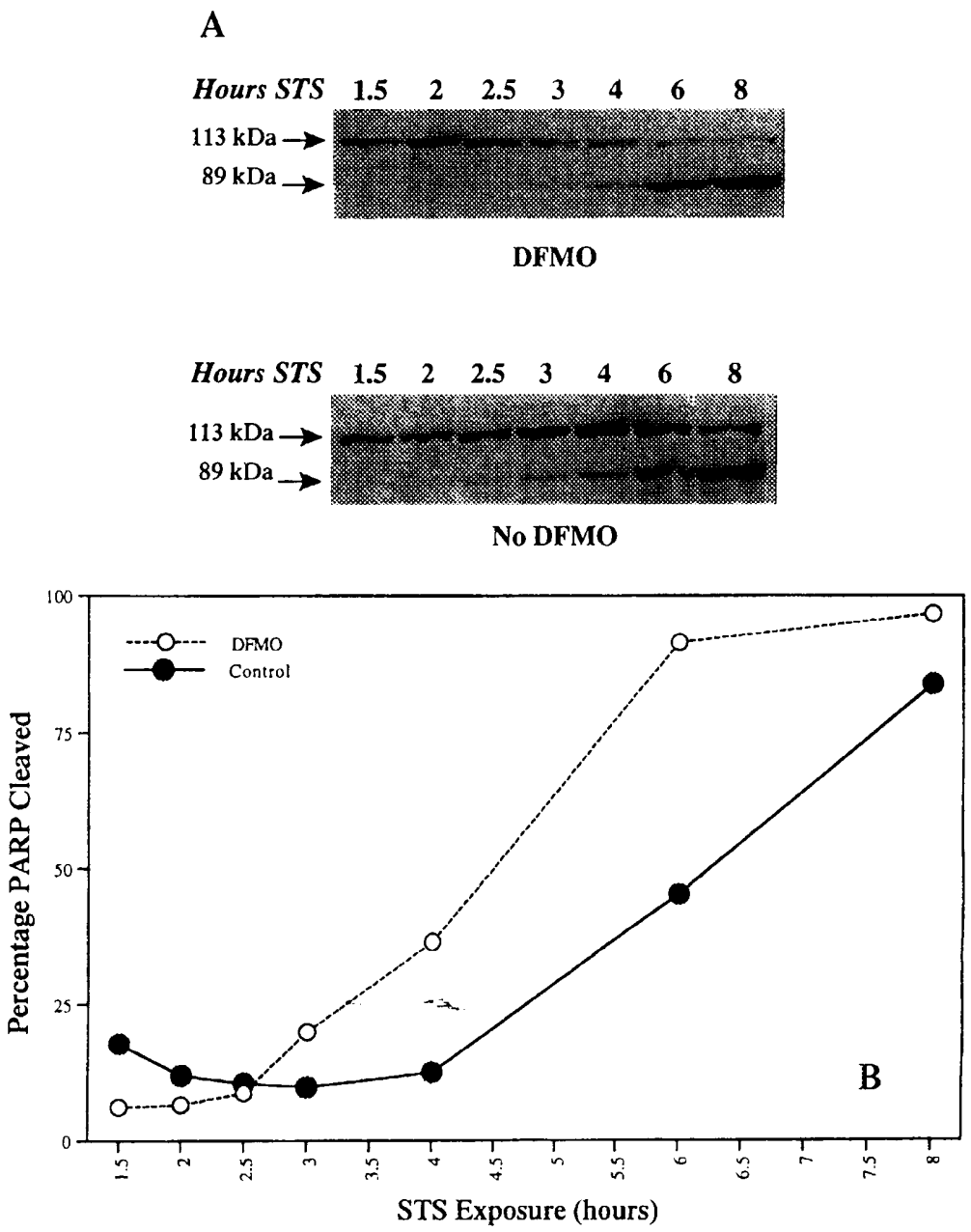



**FIG. 5.** Examination of the kinetics of apoptosis as measured by PARP cleavage. SCC-13Y cells were incubated for 8 h with 200 n*M* STS with or without pretreatment with 5 m*M* DFMO for 48 h. Panel A demonstrates the formation of PARP cleavage product at 89 kDa and the decline in the 113-kDa band. Panel B depicts the quantification of PARP cleavage as a function of exposure time. PARP cleavage is calculated as the percentage of the total signal in each lane (89 kDa + 113 kDa) that is in the 89-kDa band.

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**FIG. 4.** Detection of apoptosis by flow cytometry. Polyamine-depleted SCC-13Y and SCC-4Y cells were treated with 200 n*M* STS (8 h) ± 1 m*M* putrescine repletion. Panel A depicts representative histogram data from flow cytometry experiments for treatment groups as shown. Panel B depicts apoptosis expressed as percentage of control level, extrapolated by quantification of sub $G_1$ phase DNA, from STS-treated cells standardized to 100% with errors shown as SEM. Individual treatments with DFMO and putrescine produced levels of apoptosis identical to that of control cells within experimental error.

liferative cells, such as SCCs, may manifest a more direct correlation between polyamine depletion and induction of apoptosis, whereas more slowly proliferating cells with high $G_0$ contents may be less likely to exhibit this effect.

In our studies, polyamine depletion inhibited cell proliferation and amplified apoptosis, whereas exogenous polyamine repletion was sufficient to inhibit the DFMO-induced antiproliferative effect and amplification of apoptosis. The data on PARP cleavage indicated that polyamine depletion accelerated the kinetics of progression of apoptosis but had no influence on the overall sensitivity of the cells to STS over a broad concentration range. This finding suggests that polyamine depletion may preferentially promote apoptosis without directly affecting biochemical pathways to apoptosis. STS is a powerful inhibitor of PKC, which has been implicated as a transcriptional control element for ornithine decarboxylase, the rate-limiting enzyme of polyamine biosynthesis (*22*). While we have demonstrated that STS does not directly perturb intracellular levels of polyamines, the combination of inhibition of ornithine decarboxylase by DFMO and the transcriptional down-regulation of ornithine decarboxylase by STS may combine to influence polyamine pools and ornithine decarboxylase activity. Further studies will investigate this relationship, and whether polyamine depletion modulates the specific expression of proteins associated with apoptosis, such as TP53 and BCL2 (*1*).

We are currently examining the ability of DFMO (and other antiproliferative agents) to modulate proliferation and apoptosis in cells of a variety of human head and neck tumor cell lines. The relationship between intracellular polyamine pools and apoptosis suggests a potential relevance to clinical therapy. Agents capable of producing antiproliferative tumor growth inhibition and amplification of apoptosis may prove to be valuable in the adjuvant therapy of rapidly dividing human tumors such as those that occur in the head and neck.

## ACKNOWLEDGMENTS

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